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Single or dual experimental infections with *Vibrio aestuarianus* and OsHV-1 in diploid and triploid *Crassostrea gigas* at the spat, juvenile and adult stages

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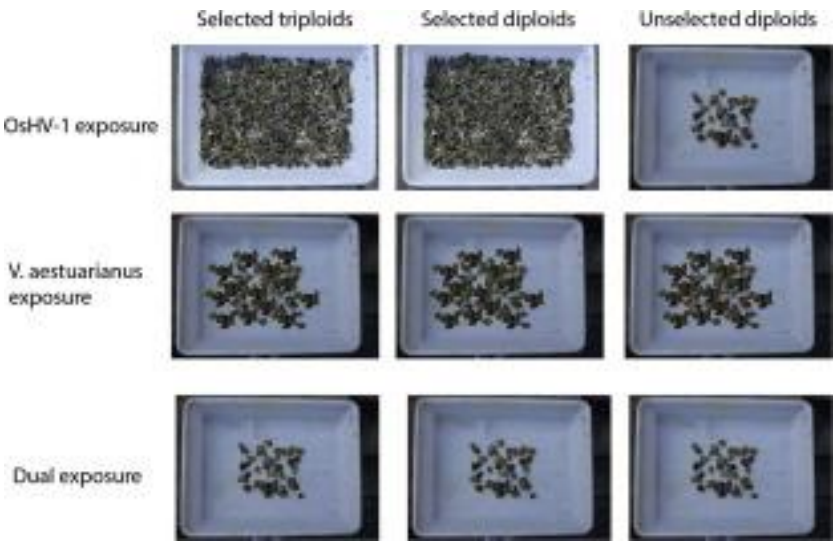
Abstract :

French production of the Pacific cupped oyster, *Crassostrea gigas*, is currently threatened by two pathogens, OsHV-1 and *V. aestuarianus*. While oysters selected for their higher resistance to OsHV-1 are now available for the industry, the impact of *V. aestuarianus* on such oysters is unknown, especially for triploids. In addition, experimental infection has used the virus or the bacteria alone, but there have been no investigations of dual exposure to these pathogens. This study is the first report of single or dual exposure in spat (Spat1 and Spat2), juvenile and adult naïve oysters. For each of the two stocks evaluated, unselected oysters and oysters selected for their higher resistance to OsHV-1 infection were tested, as well as their triploid siblings of the selected oysters produced using cytochalasin B. We confirmed that resistance to OsHV-1 infection and susceptibility to *V. aestuarianus* increased with age and size, although selected oysters were not significantly impacted by OsHV-1 whatever their ploidy, size or age. We found different mortality patterns depending on the pathogen tested. The mortality pattern was similar for oysters exposed to OsHV-1 or to both pathogens in the Spat1 trial (4 months old and 1.9 g). The mortality pattern was similar for oysters exposed to *V. aestuarianus* or to both pathogens in the Adult trial (25 months old and 63.1 g). Surprisingly, mortality was much higher (ranging from 75.9% to 100%), in particular for the selected oysters, for the Spat2 (8 months old/3.9 g) and Juvenile trials (16 months old/18.4 g) given a dual exposure, regardless of the level of selection for OsHV-1 and the ploidy state. Our findings highlight an important threat for oyster farmers: oysters exposed to both pathogens could experience dramatic mortality rates, even in oysters selected for their higher resistance to OsHV-1. Finally, our study demonstrated for the first time that triploid oysters were more susceptible to experimental challenges with *V. aestuarianus* at the spat stage than their diploid siblings. However, the difference in mortality between the triploids and diploids remained limited and ranged from 22.9% to 6.6% for spat and adults, respectively with a relatively regularly decrease in the difference with increased age.

Graphical abstract :



Highlights

► Different mortality patterns depending on the pathogen tested and the oyster life stage. ► Dual exposure lead to higher mortality than single exposure in spat and juvenile. ► Unselected and selected oysters, 2n or 3n, had very high mortality in dual exposure except in Spat1. ► Triploids had a higher susceptibility to *V. aestuarianus* than diploids.

Keywords : *Vibrio aestuarianus*, OsHV-1, Dual exposure, Ploidy, Selection, *Crassostrea gigas*

Introduction

French production of the Pacific cupped oyster, *Crassostrea gigas*, is currently threatened by two pathogens. Indeed, massive mortality events that were formerly episodic and geographically limited have occurred every year since 2008, with high mortality rates for the spat and juveniles (over 70%). A particular OsHV-1 genotype (µvar) has been identified as a causal agent implicated in the mortality (Segarra et al., 2010) which was then found during a period of *C. gigas* mortality in 2004-2005 in Normandy (Martenot et al., 2012). Moreover, significant mortality has been observed in market-sized adults since 2012 (Francois, 2015; Francois et al., 2013, 2014). The primary pathogenic agent found in the dying oysters sampled during these mortality episodes was *Vibrio aestuarianus*. Although more widespread and regular mortality have been reported in France since 2008, it is still not possible to attribute those to either a particular OsHV-1 strain or a higher virulence of *V. aestuarianus* (Goudenège et al., 2015) because these two pathogens have been described in France since 1991 and 2001 respectively (Labreuche, et al., 2006; Nicolas, et al., 1992). Genetics, pollutants, cultural practices and other factors could also be involved to explain the intense mortality caused by OsHV-1.

The co-detection of OsHV-1 with different species of *Vibrio*, including *Vibrio* belonging to the *V. splendidus* clade and *V. aestuarianus* species, has been observed during several mortality events in France (Francois et al., 2009; Lemire et al., 2015; Saulnier et al., 2010) and New Zealand (Keeling et al., 2014). Recently, mortality was reported to be higher in the presence of OsHV-1 associated with *Vibrio* species than in the presence of OsHV-1 only (Petton et al., 2015).

Several breeding investigations have confirmed the high genetic basis for survival for *C. gigas* in the spat stage (Dégremont et al., 2010) and therefore to OsHV-1 infection (Dégremont, 2011; Dégremont et al., 2015b). More recently, a mass selection breeding program lead to significant genetic progress enhancing survival and OsHV-1 resistance in *C. gigas* (Dégremont et al., 2015c). Regarding *V. aestuarianus*, one study suggested that the *Vibrio* resistance trait had a genetic basis (De Decker, 2007). Later, the genetic parameters were estimated, with a higher genetic variation from spat to adults, although heritability was weak (0.2) (Azema et al., 2015a). This study also showed no significant genetic correlation between resistance to OsHV-1 infections and resistance to *V. aestuarianus* infections. Conversely, selected oysters at the spat stage in the field seemed to exhibit dual resistance to OsHV-1 and *V. aestuarianus* experimental infections at the spat stage but not at the adult stage (Azema et al., 2015b). No experimental infection study to date has used a dual-pathogen exposure with OsHV-1 and *V. aestuarianus* even though both pathogens have been reported in numerous mortality events in France (Francois et al., 2014). A dual exposure could counteract the genetic mechanism of single disease-resistance and bypass host defenses.

The French cultured production of *C. gigas* is based on both wild-caught and hatchery-produced spat, representing 70% and 30% of the cultivated spat, respectively. This proportion varies among years due to the availability of wild-caught spat, which strongly depends on diseases and environmental conditions as well as on the number of collectors that are deployed by oyster farmers to harvest the spat. For hatchery-produced spat, production has increased three-fold during the last 10 years, reaching nearly 3 billion individual spat in 2012 (Dégremont and Benabdelmouna, 2014). This increase was primarily driven by triploid

oysters, which have a better growth and yield compared to their diploid counterparts (Dégremont et al., 2012; Hand et al., 2004). In addition, triploids are preferred over diploids in the summer because diploids are less marketable when in the spawning condition (Allen and Downing, 1986; Nell, 2002).In France, all triploid oysters are produced by private hatcheries, crossing diploid females from their own stocks with tetraploid males from a unique stock produced and maintained at the Ifremer hatchery in La Tremblade (Guo et al., 1996). Triploidy can also be induced using several methods (pressure, heat shock, chemicals) by blocking the extrusion of either the first or the second polar body that occurs during the first hour post-fertilization (Gérard et al., 1999). The induction methods offer the advantage of obtaining triploid offspring that share the same genetic background as their diploid siblings.

The effect of ploidy on pathogen-caused mortality remains unclear (Dégremont et al., 2015a). Some studies conducted in field conditions revealed that triploidy confers neither an advantage nor a disadvantage in survival based upon resistance to OsHV-1 infection in *C. gigas* (Dégremont et al., 2016) or on the abundance of *Vibrio vulnificus* and *V. parahaemolyticus* in *C. virginica* (Walton et al., 2013). In contrast, other studies found a higher disease resistance in triploids compared to diploids; for example, resistance to *Haplosporidium nelsoni* or *Perkinsus marinus* in *C. virginica* (Dégremont et al., 2012) and resistance to *Bonamia roughleyi* in *S. glomerata* (Hand et al., 1998). The effect of ploidy on disease resistance was also investigated through experimental infections by *Vibrio aestuarianus* and *V. tasmaniensis* LGP32, but inconsistent results were observed. These results were explained by different patterns of energy allocation in diploid and triploid Pacific oysters at different times of the year (De Decker et al., 2011).

Spawning is an important factor on the susceptibility to stress and mortality in diploid *C. gigas*. The energy expended during reproduction compromises the immune status of oysters, leaving them easily subject to mortality if a stress occurs in post-spawning stage, such heat stress (Li et al., 2007) or pathogens, including OsHV-1 (Dégremont et al., 2013) and *Vibrio sp* (Wendling and Wegner, 2013). Nevertheless, the partial sterility of triploids and their different energy allocations for growth and gametogenesis might not confer on them a higher resistance to stress and mortality. Furthermore, the perception of the French oyster farmer about the mortality of his stock suggests that triploids could be more sensitive to *V. aestuarianus* than diploids.

To answer this question for the French oyster industry, we designed an experiment to investigate single and dual exposure to *V. aestuarianus* and OsHV-1 in *C. gigas.* Two lines selected for their higher resistance to OsHV-1 infection, as well as their respective controls (i.e., not selected), were tested at 4, 8, 16 and 25 months old, corresponding to Spat1, Spat2, Juvenile and Adult trials respectively. To date, lines selected for their higher resistance to *V. aestuarianus* infection are not available and could not be tested in this study. In addition, and only for the selected stocks, chemically induced triploids were also evaluated using the same protocol as their diploid siblings. The main objectives of this study were to test the impact of dual disease exposure on two common genetic improvements used in oysters, selective breeding for a single disease-resistance and triploidization, for each stage of development.

1. Materials and methods

1.1. Oysters

A mass selection scheme to increase survival in *C. gigas* was performed in two stocks (named A and B) of wild oysters sampled in 2008 from two sites in the Marennes-Oléron Bay (Charente Maritime, France). For each stock, a base population, $G_{0,}$ was produced in 2009. A sub-sample was kept in our facilities to avoid disease-related mortality and to produce the control line of the following generation (G_1-C). The other sub-sample of oysters was deployed in the field, where mortality outbreaks caused by OsHV-1 have been routinely observed each year since 2009 (Dégremont et al., 2015c). The survivors were spawned in 2010 to produce the selected line G_1-S. The same approach was used in February 2011, March 2012 and March 2013 to produce G_2, G_3 and $G_{4,}$ respectively; further details are given in Dégremont et al. (2015c).

In our study, we used the selected and the control lines of each stock, A and B, produced for G_4 in 2013. The selected lines, named AS and BS, respectively, for stock A and B, and the control lines (named AC and BC) were all diploids. Twenty minutes post-fertilization of the eggs with the spermatozoa for AS and BS, half of the embryos were treated with cytochalasin B for 15 min to suppress the expulsion of the second polar body (PB2) according to a modified protocol (Dégremont et al., 2016; Gérard et al., 1999). The resulting triploid embryos were named 3nAS and 3nBS, which are siblings of AS and BS, respectively. The DNA ploidy level of each triploid line was verified using flow cytometry (FCM) from a sample of larvae as previously described (Dégremont et al., 2016). To our knowledge, this approach doesn't produce tetraploid larvae and spat but it is rather well known to produce a majority of triploids, small amount of diploids and some rare aneuploids. Our protocol used additional steps of sorting by size of larvae and spat in combination with extensive flow cytometry analyses to constitute pure triploid batches with undetectable proportions of spat of unwanted ploidy levels.

1.2. Viral and bacterial suspension

The *V. aestuarianus* strain used in the bacterial challenges was the highly pathogenic strain 02/041 that was isolated during a mortality episode in adults and has been previously studied (De Decker and Saulnier, 2011; Garnier et al., 2007). The *Vibrio* suspension was obtained from an isolate maintained at -80°C. The bacterial strain was placed in liquid Zobell and incubated for 24 h at 20°C with constant shaking at 20 rpm. The resulting solution was centrifuged at 3200 x g for 10 min. The supernatant was discarded, and the pellet was washed and suspended in sterile artificial sea water (SASW) before adjustment to $OD_{600nm} = 1$. Purity and bacterial concentration were determined by plating on Zobell agar.

The viral suspension was obtained using the protocol of Schikorski et al. (2011). Briefly, after being experimentally infected by injecting 50 µl of an OsHV-1 µvar suspension, dead oysters were dissected. The mantle and gills were removed, pooled, diluted, crushed and filtered using a 0.22-µm filter to obtain a clarified tissue homogenate. The absence of bacteria was confirmed by plating on Zobell agar at 20°C. The viral suspension was maintained at 4°C and renewed when necessary.

1.3. Mortality induction protocols

All lines (AC, AS, 3nAS for the stock A; BC, BS and 3nBS for the stock B) were evaluated for resistance to either OsHV-1 or *V. aestuarianus* and to both pathogen infections under experimental infections in four trials: Spat1, Spat2, Juvenile and Adult, for which oysters were 6, 9, 15 and 25 months old, respectively, and weighing about 1.9, 3.9, 18.4 and 63.1 g, respectively (Table 1). The stage name used in our study referred to the stage used by the French oyster farmers which is related to the size of the oysters (spat<5g, juvenile 5-25g, adult>25g). For each pathogen exposure (OsHV-1, *V. aestuarianus* and both), as well as for one control, three 10-L tanks were used for the Spat1, Spat2 and Juvenile groups, and 6 tanks for the Adult group, each containing the six lines. The density per line and per tank was 25 oysters for Spat1 and Spat2, which was reduced to 15 for Juveniles and 5 for Adults, to maintain a reasonable biomass in each tank (Table 1). All tanks were filled with filtered and UV-treated seawater and maintained at 21°C with adequate aeration and without adding food. The salinity averaged 32‰ for all challenges. Each oyster was individually tagged for identification.

A cohabitation experimental protocol was adapted to evaluate disease resistance in *C. gigas*. First, naïve oysters were anaesthetized (Suquet et al., 2009) and injected with 50 µL of infectious suspensions (bacterial or viral suspension) into the adductor muscle using a 1 mL micro-syringe equipped with an 18-g needle. The injected oysters were transferred into 10-L tanks for 24 h. In the second step, they were placed for 48 h in contact with the 6 lines to test their disease resistance. The control tank used the same protocol but with naïve oysters injected with SASW and placed in contact with the 6 lines. Approximately 50 g of injected oysters (n=4) were placed in each 10-L tank. For dual infection, an equivalent weight of oysters injected with viral suspension (n=2) and oysters injected with bacterial suspension (n=2) was simultaneously placed in the tanks. We considered a dead oyster as an animal that was unable to close its valves after 5 min out of the water. Trials lasted 15 days for Spat1, Spat2, and Juvenile and one month for Adult.

1.4. Detection of OsHV-1 and V. aestuarianus DNA

For the Juvenile and Adult trials, moribund oysters of all lines were sampled for the detection of OsHV-1 and *V. aestuarianus* DNA. Total DNA was extracted from tissue fragments (mantle + gills) using the QIAgen (Hilden, Germany) QIAamp tissue mini kit combined with the use of the QIAcube automated sample preparation system according to the manufacturer's protocol. The total DNA amount was adjusted to 5 ng/µl following NanoDrop (Thermo Scientific, Waltham, USA) measurement.

A real-time PCR assay was conducted on MX3000 and MX3005 Thermocyclers (Agilent, Santa Clara, USA) using the Brilliant III Ultrafast kit (Stratagene). Each reaction was run in duplicate in a final volume of 20 µl containing a DNA sample (5 µl at 5 ng/µl), 200 nM of each primer (for OsHV-1: DPF 5' ATT GAT GATGTG GAT AAT CTG TG 3' and DPR 5' GGT AAA TAC CAT TGG TCT TGTTCC 3' (Webb et al., 2007); for *V. aestuarianus*: DNAj-F 5' GTATGAAATTTTAACTGACCCACAA3'; and DNAj-R 5' CAATTTCTTTCGAACAACCAC 3' (Saulnier et al., 2009)) and 200 nM of oligonucleotide probe (for *V. aestuarianus* DNAj probe 5' TGGTAGCGCAGACTTCGGCGAC). Real-time

PCR cycling conditions were as follows: 3 min at 95°C, followed by 40 cycles of amplification at 95°C for 5 s and 60°C for 20 s. The standard curve was determined on serially diluted titrated plasmids provided by the National Reference Laboratory (Ifremer La Tremblade). For OsHV-1 DNA quantification, melting curves were also plotted (55-95°C) to ensure that a single PCR product was amplified for each set of primers. Negative controls (without DNA) were included.

1.5. Statistical analyses

Mortality was analyzed using the Genmod procedure with the SAS 9 software (SAS Institute Inc., 2012) by a logistic regression for binomial data.

To compare the selection factor, only mortality data from the diploid lines AS, BS, AC and BC were analyzed for each trial (Spat1, Spat2, Juvenile and Adult) and for each experimental infection condition (OsHV-1, *V. aestuarianus* and both pathogens) using the following model:

$$Y_{ij} = Logit(\pi) = \ln\frac{\pi}{1-\pi} = \mu + stock_i + selection_j + stock_i * selection_j + \varepsilon$$

where Y_{ij} was the mortality probability, μ was the intercept, $stock_i$ represented stock A or stock B, and $selection_j$ represented the level of selection for OsHV-1 resistance (selected S or control C).

To compare the ploidy factor, only mortality data from AS, BS, 3nAS and 3nBS were analyzed for each trial (Spat1, Spat2, Juvenile and Adult) and each experimental infection condition (OsHV-1, *V. aestuarianus* and both pathogens) using the following model:

$$Y_{ij} = Logit(\pi) = \ln\frac{\pi}{1-\pi} = \mu + stock_i + ploidy_j + stock_i * ploidy_j + \varepsilon$$

where Y_{ij} was the mortality probability, μ was the intercept, $stock_i$ represented stock A or stock B, and $ploidy_j$ represented the ploidy of the lines (diploid or triploid).

In addition, mortality among the experimental infection conditions within a trial was analyzed either for the selection factor or the ploidy factor using the following model:

$$Y_i = Logit(\pi) = \ln\frac{\pi}{1-\pi} = \mu + experimental\ infection_i + \varepsilon$$

where Y_i was the mortality probability, μ was the intercept, and $experimental\ infection_i$ represented the pathogens used (OsHV-1, *V. aestuarianus*, and both pathogens)

Results

No mortality occurred in the control tanks for all experimental infection trials, and all mortality data for each line are provided in supplementary tables 1 & 2.

1.6. Comparison of the mortality between diploid unselected (AC and BC) and diploid selected (AS and BS) oysters for their higher resistance to OsHV-1 infection

1.6.1. Experimental infections by OsHV-1

For the experimental infection by OsHV-1 only, mortality started three days post-infection for the unselected oysters (AC/BC) and lasted one week. The mean final cumulative mortality among the tanks of the unselected oysters reached 90.2% in the Spat1 trial and was significantly higher than the selected oysters (AS/BS) at 33.6% ($p<0.0001$) (Fig. 1A) (Table 2). Similar findings were observed for the Spat2 and Juvenile trials, although the mortality of the unselected oysters decreased to 79.3% and 60.3%, respectively, and to a lesser extent for the selected oysters, to 6.1% and 14.7% (Fig. 1A). For the Adult trial, the mortality of the unselected oysters (17.5%) was not significantly higher than the mortality of the selected oysters (1.7%) ($p=0.48$) (Table 2) (Fig. 1A). All other factors tested were not significant with the exception of the tank factor for the Spat2 ($p=0.04$) and Juvenile ($p=0.02$) (Table 2) trials.

1.6.2. Experimental infections by V. aestuarianus

The onset of mortality was 5 days post-infection, and a peak was observed 5 to 10 days later. For Spat1, the final cumulative mortality reached 15.6% for the unselected oysters (AC/BC) and was significantly higher than the mortality for the selected oysters (AS/BS) (4.7%) ($p<0.01$) (Fig. 1B). All other factors were not significant with the exception of the tank factor ($p<0.01$) (Table 2). Similar results were observed in the Spat2 trial, although mortality was higher, at 47.9% and 34.6% for the unselected and selected oysters, respectively (Fig. 1B) (Table 2). This trend was confirmed in the Juvenile trial, with mortality rising to 68.3% and 64.2% for unselected and selected oysters and reaching 75.6% and 76.7%, respectively, for the Adult trial (Fig. 1B). None of the other factors was significant with the exception of the tank factor in Juvenile trial ($p<0.01$) (Table 2).

1.6.3. Experimental infections by OsHV-1 and V. aestuarianus

For the experimental infection involving the two pathogens, the onset of mortality was 5 days post-infection, and a peak was observed 5 to 10 days later for the Spat2, Juvenile and Adult trials, while mortality was faster for Spat1. For Spat1, the mean final cumulative mortality reached 90.2% for the unselected oysters (AC/BC) and 22.8% for the selected oysters (AS/BS) (Fig. 1C). A significant difference in mortality was found between the AS/BS and AC/BC. In contrast, mortality increased for both groups, to 98.7% for the unselected oysters and 75.9% for the selected oysters for Spat2 and was not significantly different ($p=0.96$) (Fig. 1C) (Table 2). Mortality was high and not significantly different in juvenile trial in

unselected (100%) and selected oysters (90%) (Fig. 1C) (Table 2). Lower mortality was reported for the Adult trial, but it still remained high, at 60.0% and 76.3%, for the unselected and selected oysters, respectively (Fig. 1C). None of the factors was significant for the Adult trial except the selection factor with lower mortality for the unselected oysters (p=0.03) (Table 2).

1.6.4. Comparison of mortality among single or dual disease exposure

For Spat1, the final cumulative mortality among the lines was significantly different among the disease conditions tested (p<0.0001), with the lowest mortality for the exposure to *V. aestuarianus* (10.1%), and similar mortality for the oysters exposed either to OsHV-1 (61.9%) or both diseases (56.5%). For the Spat2 and Juvenile trials, the dual exposure to pathogens involved significantly higher mortality (87.3% and 95.0%, respectively) than single disease exposure (p<0.0001). In a closer look, the unselected oysters in the Spat2 trial exposed to OsHV-1 had higher mortality (79.3%) than those exposed to *V. aestuarianus* (47.9%), while it was the opposite for the selected oysters, at 6.1% and 34.6%, when exposed to the virus and the bacteria, respectively (Fig. 1AB). For Juveniles, unselected oysters had similar mortality when exposed either to OsHV-1 (60.3%) or *V. aestuarianus* (68.3%), while the selected oysters had significantly higher mortality when exposed to the bacteria (64.2%) in comparison to OsHV-1 (14.7%) (Fig. 1AB). For Adults, the final cumulative mortality among the lines was significantly different among the disease conditions tested (p<0.0001), with the lowest mortality for the exposure to OsHV-1 (8.0%), and similar mortality for the oysters exposed either to *V. aestuarianus* (76.2%) or both diseases (70.5%).

1.7. Comparison of the mortality between diploid (AS/BS) and triploid (3nAS/3nBS) oysters using lines selected for their higher resistance to OsHV-1 infection

1.7.1. Experimental infections by OsHV-1

For the experimental infections with OsHV-1 only, the onset of mortality was 5 days post-infection and no peak of mortality was observed. The mean final cumulative mortality among lines (AS, BS, 3nAS, 3nBS) remained low, at 32.1% for Spat1, 7.9% for Spat2, 13.2% for Juveniles and 2.5% for Adults. For each trial, the tank factor was not significant. The interaction between ploidy and stock was significant for the Spat 1 trial (p=0.02) (Table 3). At the stock level, triploid oysters (3nAS) (24.5%) had significantly lower mortality than diploid oysters (AS) (40.0%) for stock A (p=0.02), while it was the opposite for stock B (<0.0001), with 27.2% for diploids (BS) and 36.6% for triploids (3nBS) (Fig. 2A). For Spat2, Juvenile and Adult trials, mortality was not significantly different between diploid (6.1%, 14.7% and 1.7% for Spat2, Juvenile and Adult, respectively) and triploid (9.7%, 11.7 and 3.3%) as well as between stocks (Fig. 2A) (Table 3).

1.7.2. Experimental infections by V. aestuarianus

For the experimental infections with *V. aestuarianus* only, the onset of the mortality was 5 days post-infection, and a peak was observed 5 to 10 days later. The mean final cumulative mortality among the lines increased from 16.2% for Spat1 to 41.7% for Spat2, 69.0% for Juveniles and 80.0% for Adults. For each trial, the tank factor was significant for the Spat1, Spat2 and Juvenile (Table 3). In contrast, the interaction between ploidy and stock was not significant (Table 3). For all trials, mortality was similar between the two stocks, with the exception of Juveniles, for which stock A (78.9%) had significant higher mortality than line B (59.2%)(Table 3). In contrast, triploids (3nAS/3nBS) had a significantly higher mortality than their diploid counterpart (AS/BS), at 27.6% and 4.7%, respectively, in Spat1 and 48.9% and 34.6% in Spat2 (Fig. 2B). Finally, final cumulative mortality was not significantly different between triploids (73.9% and 83.3%) and diploids (64.2% and 76.7%) in Juveniles and Adults, respectively (Fig. 2B) (Table 3).

1.7.3. Experimental infections by OsHV-1 and V. aestuarianus

For the experimental infection involving the two pathogens, the onset of mortality was 5 days post-infection, and a peak was observed 5 to 10 days later. The mean final cumulative mortality among the lines was the lowest for Spat1 (22.5%) and high for Spat2 (82.2%), Juveniles (93.3%) and Adults (83.3%). Within trials, the replicate factor was significant for Spat1 and Spat 2, but not for Juveniles and Adults (Table 3). Mortality was not significantly different between the two stocks for each trial, as well as the interaction between stock and ploidy (Table 3). The final mean cumulative mortality was not significantly different between ploidy for Spat1, Juveniles and Adults, with 22.8% and 22.3% for diploid (AS/BS) and triploid (3nAS/3nBS), respectively, for Spat1, 90.0% and 96.7% for Juveniles, and 78.3% and 88.3% for Adults (Table 3) (Fig. 2C). In contrast, triploids (88.5%) had a higher mortality than diploids (75.9%) in Spat2 ($p<0.01$) (Table 3) (Fig. 2C).

1.7.4. Comparison of mortality among single or dual disease exposure

For Spat1, the final cumulative mortality was significantly different among the disease conditions tested ($p=0.04$), with the lowest mortality for the exposure to *V. aestuarianus* (16.2%), intermediate when the two diseases were involved (22.5%) and the highest for the exposure to OsHV-1 (32.1%). For Spat2 and Juvenile trials, the dual exposure to diseases involved significantly higher mortality (82.2% and 93.3%, respectively) than single disease exposure ($p<0.0001$), with 41.7% and 69.0% for *V. aestuarianus*, respectively, and 7.9% and 13.2% for OsHV-1, respectively. Finally, exposure to *V. aestuarianus* or exposure to the bacteria and OsHV-1 involved high but similar mortalities, of 80.0% and 83.3%, respectively, which were significantly higher than the mortality observed when oysters were exposed to OsHV-1 only (2.5%) ($p<0.0001$).

1.8. Quantification and detection of OsHV-1 and V. aestuarianus

For both Juvenile and Adult trials, all moribund oysters exposed to OsHV-1 were detected as positive, with a high amount of viral DNA $>10^{+7}$ copies per mg of fresh oyster tissue (Table 4). However, 33% and 66% also tested positive for *V. aestuarianus*, but at a lower amount ($7.7\ 10^{+2}$ and $1.2\ 10^{+5}$ copies of bacterial DNA per mg of tissues for Juveniles and Adults, respectively), suggesting a cross contamination.

After exposure to *V. aestuarianus*, this pathogen was detected in 96% and 100% of the moribund oysters analyzed, ranging from $1.2\ 10^{+6}$ to $1.1\ 10^{+8}$ copies of bacterial DNA per mg of fresh oyster tissue (Table 4). OsHV-1 was also detected in 40 and 54% of Juveniles and Adults, respectively, but a low level, with $1.0\ 10^{+3}$ and $3.9\ 10^{+3}$ copies per mg of tissues, respectively. Again, this suggested a cross contamination.

For the dual exposure experiments, 82 and 89% were positive for OsHV-1 in Juveniles and Adults, respectively, with a high amount of DNA virus, exceeding 10^{+7} (Table 4). Similarly, all oysters also tested positive to *V. aestuarianus* and again at a high amount, with $4.8\ 10^{+5}$ and $7.1\ 10^{+7}$ DNA copies per mg of fresh oyster tissue (Table 4). Regardless of the stock (A or B), the level of selection (unselected or selected for higher resistance to OsHV-1) and the ploidy (diploid or triploid), most of the individuals tested had very high DNA quantities of both pathogens (Fig. 3AB).

2. Discussion

Three main findings were revealed in this study. The first concerned different mortality patterns among pathogen exposure and among stages (spat, juvenile and adult), the second was the consequence of the dual disease exposure on oysters selected for their higher resistance to OsHV-1, and the third reveals for the first time the impact of the ploidy level on disease-resistance for each and both diseases tested.

Field grow-out means that oysters are continuously exposed to pathogens present in the environment. Nevertheless, OsHV-1 is mostly reported in *C. gigas* spat during mortality events in France, while it is *V. aestuarianus* in adults (Barbosa Solomieu et al., 2015). Our experimental infections reinforced this statement, as previously demonstrated by Azema et al.(2015a), with oysters more susceptible to the virus than the bacteria at the spat stage, while it was the opposite in adults regardless of the lines tested (Fig. 1AB & 2AB) .

Our study confirms that oysters selected for their higher resistance to OsHV-1 had significantly lower mortality than unselected oysters from spat to adults when they were exposed to the virus only (Fig. 1A). The difference in mortality between the unselected and selected oysters decreased from Spat1 to Adult, confirming the development of the resistance to OsHV-1 infection with age and size for the unselected oysters (Dégremont, 2013). In addition, our study confirmed that selecting for resistance to OsHV-1 infection in the spat stage did not confer either higher resistance or susceptibility to *V. aestuarianus* infection in adults (Fig. 1B), which is in agreement with a recent study (Azema et al., 2015b). Such findings have been commonly reported for disease-resistant oysters when tested against another disease for which they were not selected, underlying the absence of significant positive or negative genetic correlations between the resistances to two pathogens (Burreson, 1991; Dove et al., 2013; Frank-Lawale et al., 2014; Gay, 2004; Gomez-Leon J et al., 2008).

Dual exposure to the bacterium and the virus in Spat2 and Juveniles using 8-month-old to 16-month-old and 3.9 and 18.4 g oysters revealed a significant increase in mortality in comparison with single disease exposure, either by OsHV-1 or by *V. aestuarianus*. This finding was previously observed with other experimental infections, which induced higher mortalities when injecting a mix of *Vibrio* strains (Gay et al., 2004). This result could be due to increased virulence linked to bacterial cooperation (De Decker et al., 2013; Lemire et al., 2015). Moreover, it was observed in juvenile oysters that a rapid *Vibrio* spp. colonization followed by viral replication precedes oyster death (Petton et al., 2015). In our study, we can also hypothesize a direct cooperation between OsHV-1 and *V. aestuarianus* resulting in an increased virulence, as documented for human herpes viruses and bacteria (Slot, 2002) or human respiratory diseases involving influenza virus and bacteria (Smith and Sweet, 2002). Viruses can aid bacteria in all aspects of their pathogenesis (penetration, growth, interference with host immune response, and causation of damage to the host), and conversely bacterial proteases may help the influenza virus to infect cells. We can also propose that the two pathogens act independently but led to additive affects at the animal level (individual co-infection) or the population level. Interestingly, whatever the molecular mechanisms, additive or cooperative effects of those two pathogens were

observed in Spat2 and Juvenile oysters only. Either these developmental stages or the physiological status of those animals can be particularly permissive to those two organisms. Indeed, reproductive status may have increased the susceptibility of the oysters tested to pathogens, as previously reported in *C. gigas* for OsHV-1, *Vibrio tasmaniensis* and *V. aestuarianus* (De Decker et al., 2011; Dégremont et al., 2016; Li et al., 2007; Samain et al., 2007; Wendling and Wegner, 2013). Our experimental oysters were produced in February 2013, and might not have experienced a first spawning event in the Spat1 stage during June 2013 due to their young age and their small size, while they might have spawned in Spat2 (October 2013) and Juvenile (June 2014) stages. Biologically speaking, spat and juveniles would be unable to reproduce, but here we referred to the stages used by the French oyster farmers which graded their oysters by size. In addition, the last trial of Adults was undertaken in March, which corresponds to the sexual initiation of gametogenesis when the water temperature is low (Fabioux et al., 2005). Unfortunately, we did not control the ripeness of the oysters before our experimental infection. Such control should be systematically undertaken in future experiments.

The novelty of our study about the dual exposure concerned in particular the selected oysters for their higher resistance to OsHV-1 infection. Selection to improve the resistance to OsHV-1 infection in *C. gigas* can be easily achieved throughout selective breeding programs (Dégremont et al., 2015b; Dégremont et al., 2015c). In the future, oysters selected for their higher resistance to OsHV-1 infection should be used more frequently by oyster farmers, as commercial hatcheries have been developing their own selected lines. Similarly, wild oyster populations of *C. gigas* subsequently exposed to several generations of selective disease pressure should develop a higher resistance to OsHV-1 infection, as observed for *Bonamia ostreae* in *Ostrea edulis* in the USA (Elston et al., 1987). Nevertheless, the higher mortality for dual exposure than single exposure obviously had a greater effect on the selected oysters (AS/BS/3nAS/3nBS), and to a lesser extent the unselected oysters (AC/BC) (Fig. 1ABC & 2ABC). We noted that regardless of the stock (A or B), the level of ploidy (2n or 3n) and the level of selection (unselected or selected for higher resistance to OsHV-1), most of the individuals were highly infected by both pathogens in the Juvenile and Adult stages during the dual exposure (Fig. 3AB). Thus, such exposure counteracted the genetic progress for the resistance to OsHV-1 and bypassed the defense of the host against the virus. Consequently, selection to improve OsHV-1 resistance could be irrelevant when those oysters are exposed to both the virus and the bacteria, although a small difference in mortality in favor of the selected oysters remained (10-13%) (Fig. 1C). The underlying mechanisms of such mortality are unknown and further investigations should address this point. Meanwhile, such mortality seems not to occur in the field, as oysters selected for their higher resistance to OsHV-1 have always exhibited higher survival than unselected oysters each year since 2001 in the Marennes-Oléron Bay (Dégremont et al., 2010; Dégremont et al., 2015c). Nevertheless, our findings highlighted an important threat for oyster farmers. Thus, selective breeding programs should focus on dual resistance, which should be possible according to a recent study showing no significant genetic correlation between the resistance to OsHV-1 and the resistance to *V. aestuarianus* (Azema et al. 2015a).

Triploid oysters grown by the farmers are mostly all-triploid oysters produced by mating diploid females to tetraploid males (Guo et al., 1996). Commercial hatcheries used their own diploid stocks while the tetraploid oysters come from a unique stock produced by the patented method of

Benabdelmouna and Ledu (2007). In our study, the chemically induced triploids have the main advantage of not confounding the ploidy and the germplasm factors. Selected triploids performed as well as their diploid siblings when exposed to OsHV-1, whatever their age or size, in experimental infection under laboratory conditions. This confirmed results obtained in field conditions that OsHV-1 resistance was not substantially altered by triploidization and that mortality related to OsHV-1 is similar between diploids and triploids in *C. gigas* when the same germplasm is used for both ploidy states (Dégremont et al., 2016).

As observed for the selected diploids, the susceptibility of triploids to *V. aestuarianus* increased with size and age (Fig. 2A). Interestingly, our study clearly showed that triploid oysters seemed more susceptible to *V. aestuarianus* than their diploid siblings, particularly at the spat stage. This is the first report in the literature. Nevertheless, the difference in mortality between the two ploidy states remained limited and regularly decreased from Spat1 to Adult, with 22.9%, 14.6%, 9.7% and 6.6%, respectively. One of the hypotheses to explain the higher susceptibility of triploids would be a higher risk of infection by the bacteria due to their higher filtration rate. This statement is speculative only because no studies, surprisingly, have compared the filtration rate between diploid and triploid oysters. A second hypothesis considers reproductive effort. Despite much lower mature gamete production in triploid oysters relative to diploid, their reproductive effort can be significant, even in young individuals (Normand et al., 2009). In addition, a study revealed that active gametogenesis periods correspond to higher susceptibility to vibriosis and that there is a significant interaction of the season with ploidy (De Decker et al., 2011). In our study, such interaction was not reported because triploids had always higher mortality than diploids (Fig. 2B) throughout the four trials.

Although, the chemically induced triploids showed a higher susceptibility to *V. aestuarianus*, than their diploid siblings, further investigations are now necessary to focus on all-triploids used by farmers.

The difference in mortality among tanks observed, in particular when *V. aestuarianus* was involved (Table 2) could be explained by the mortality induction protocols used, which may not be identical among the tanks. Naïve oysters were injected with 50 µL of infectious suspensions (bacterial or viral suspension) into the adductor muscle and then placed for 48 h in contact with the 6 lines to test their disease resistance. If the injected oysters excrete bacteria or virus in a limited amount (< to the minimal infective dose) during this period, we failed to trigger the mortality in one tank, as observed for one tank of *V. aestuarianus* in spat2. Conversely, if the injected oysters excreted high quantities of bacteria or virus, one tank had much higher mortality for the others as observed for *V. aestuarianus* in Juvenile. Thus, the donors combined to the genetic resistance of oysters could explain the difference observed among tank, although the ranking of the batches evaluated were mostly identical among the tanks.

Finally, the detection at a low level of OsHV-1 DNA for the bacterial challenge and *V. aestuarianus* DNA for the viral challenge for some of the moribund oysters is more likely to be related to degraded DNA due to the UV-treated seawater. Their detection could be explained by the seawater pumped into the Marennes-Oléron Bay, where mortality related to OsHV-1/*V. aestuarianus* is now commonly reported. Thus, filtration and UV-treated seawater likely

removed most bacteria and viral particles, and the DNA detected was likely inactive or degraded DNA. The absence of mortality for controls comfirmed this statement.

3. Conclusions

This study is the first to investigate the mortality of a single or a dual exposure to the two main disease agents affecting the French oyster production of *C. gigas*. Although only two stocks were used in our study, the oysters also reflected different source of oysters used by farmers with unselected oysters, oysters selected for their higher resistance to OsHV-1, and their selected triploid siblings. The experimental infections conducted from spat to adult using naïve oysters clearly revealed different mortality patterns depending on the pathogen tested. Thus, resistance to OsHV-1 infection and susceptibility to *V. aestuarianus* increased with age and size, although selected oysters were not significantly impacted by OSHV-1 whatever their size or age. We clearly showed that the mortality pattern was similar when oysters were exposed to OsHV-1 or exposed to both pathogens for Spat 1 (4 months old and 1.9 g). Similarly, the mortality pattern was similar when oysters were exposed to *V. aestuarianus* or exposed to both pathogens in Adults (25 months old and 63.1 g). Surprisingly, mortality was much higher for Spat2 and Juveniles (8-16 months old and 3.9-18.4 g) for the dual exposure than a single pathogen exposure, regardless of the level of selection for OsHV-1 and the ploidy. Our finding highlighted an important threat for oyster farmers, where oysters exposed to both pathogens at the same time could experience dramatic mortality, even in oysters selected for their higher resistance to OsHV-1. Finally, our study reports for the first time a potential impact of triploidy on the susceptibility to *V. aestuarianus*. Tested triploid oysters were more susceptible to *V. aestuarianus* than their diploid siblings, although the difference in mortality between both ploidy states remained limited and regularly decreased from Spat1 to Adults, at 22.9% and 6.6%, respectively. Our study highlighted the importance of developing rapid selective breeding programs for dual resistance to OsHV-1 and *V. aestuarianus* infections.

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Author contributions

Conceived and designed the experiments: PA, MAT and LD. Production of the animals: PA, AB, LD. Performed the experiments: PA. Analyzed the data: PA and LD. Wrote the manuscript: PA, MAT, AB and LD. All authors read and approved the final version of the manuscript.

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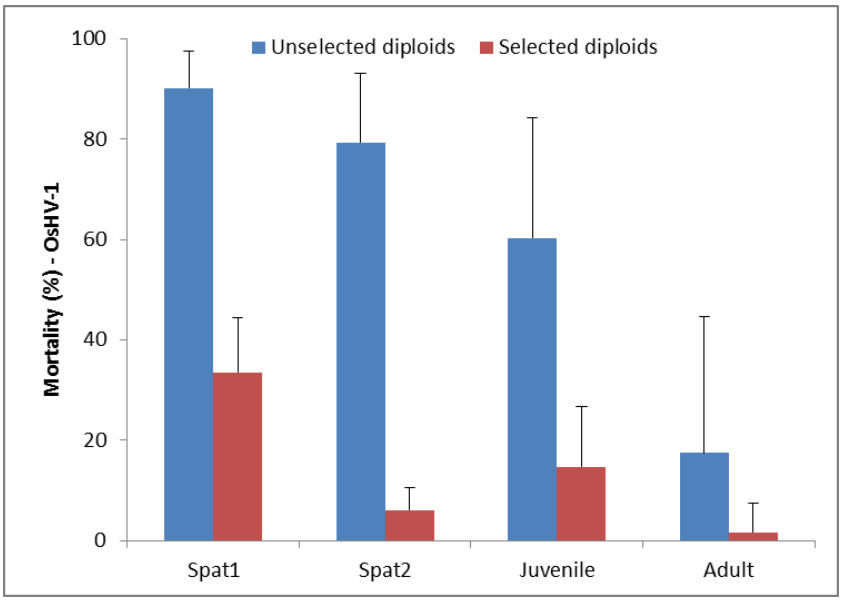
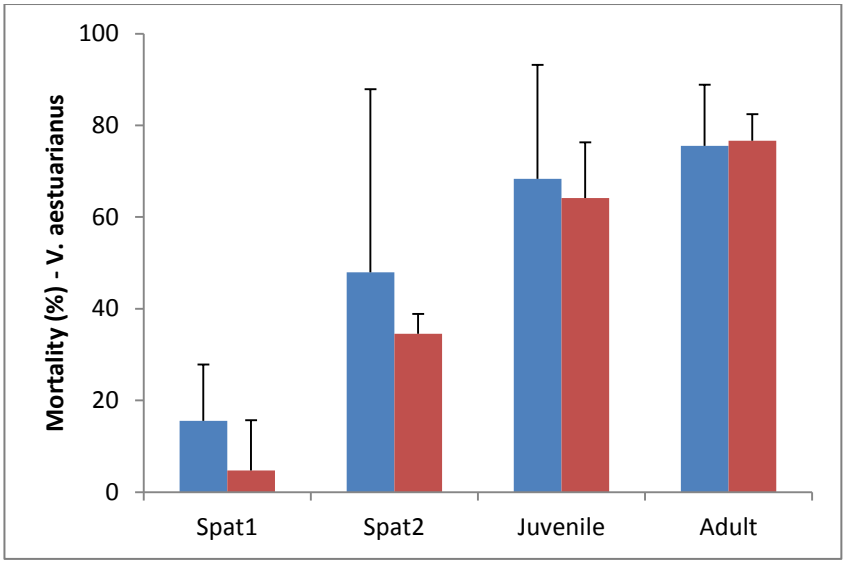
695

696

A


Unselected diploids
Selected diploids
Mortality (%) - OsHV-1
100
80
60
40
20
0
Spat1
Spat2
Juvenile
Adult

B


Mortality (%) - V. aestuarianus
100
80
60
40
20
0
Spat1
Spat2
Juvenile
Adult

C

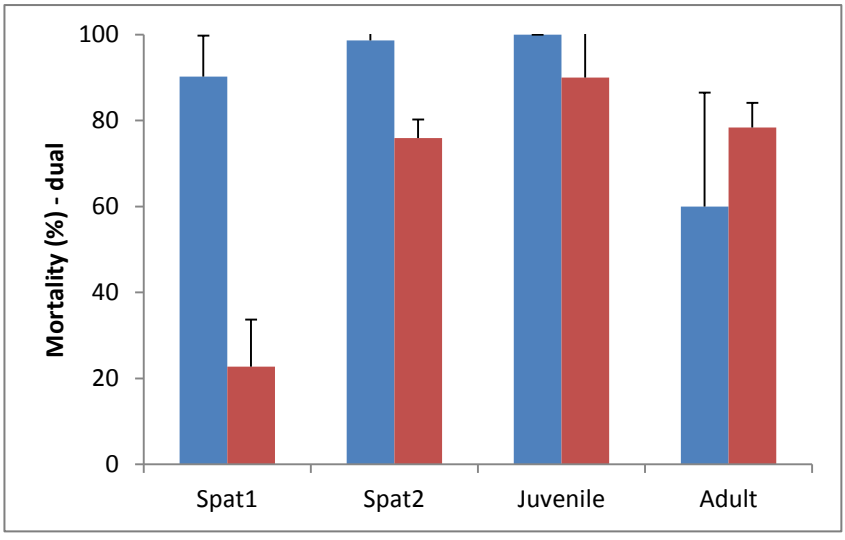
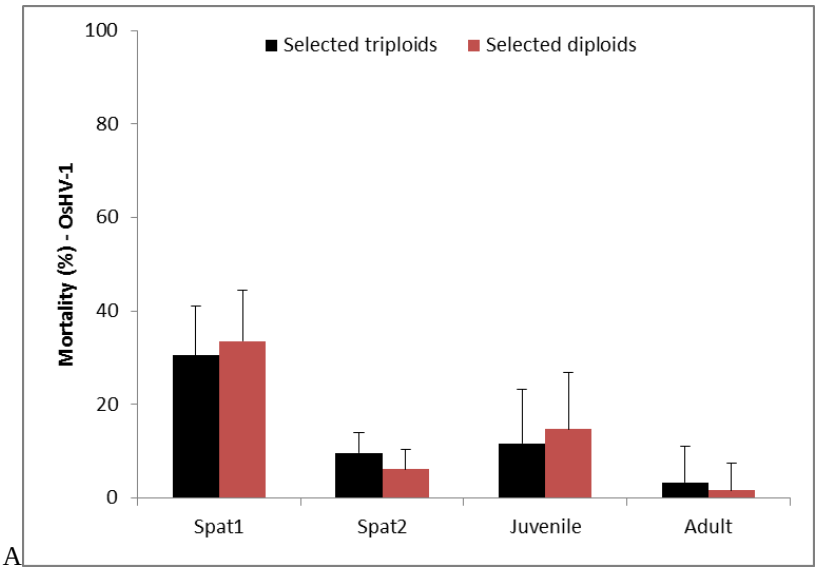
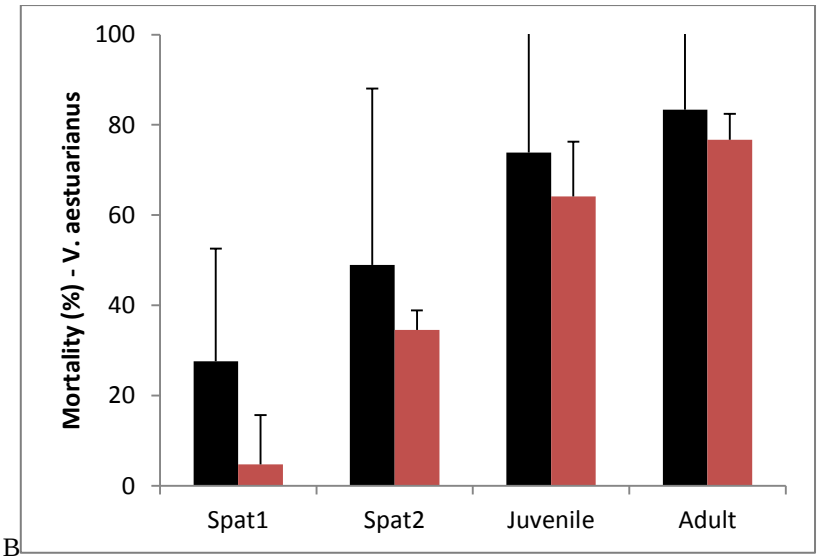

Mortality (%) - dual
100
80
60
40
20
0
Spat1
Spat2
Juvenile
Adult

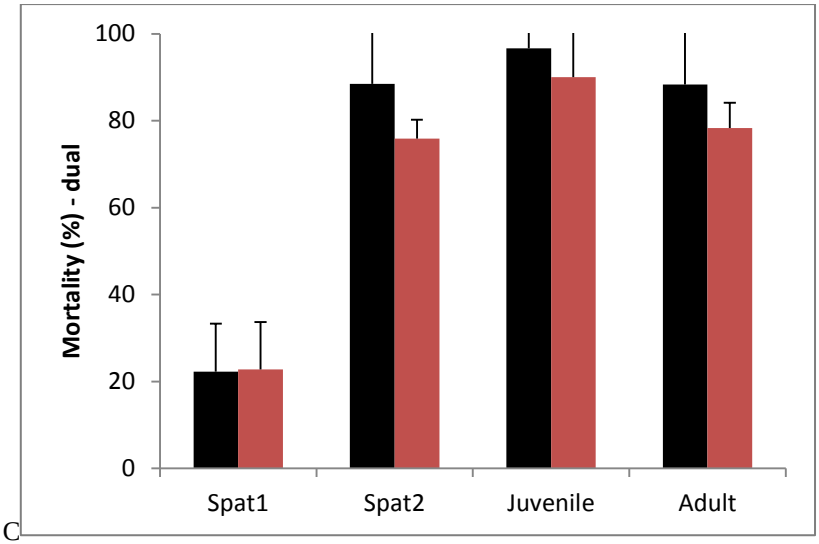
Fig. 1: Mean final mortality (+SD between stocks A and B) for unselected and selected diploid oysters after either single pathogen exposure to OsHV-1 (A) and *V. aestuarianus* (B) or dual pathogen exposure (C). Trials lasted 15 days for Spat1, Spat2 and Juvenile and one month for Adult. See Table 1 for more details of each trial.


Selected triploids
Selected diploids
Mortality (%) - OsHV-1
100
80
60
40
20
0
Spat1
Spat2
Juvenile
Adult

A

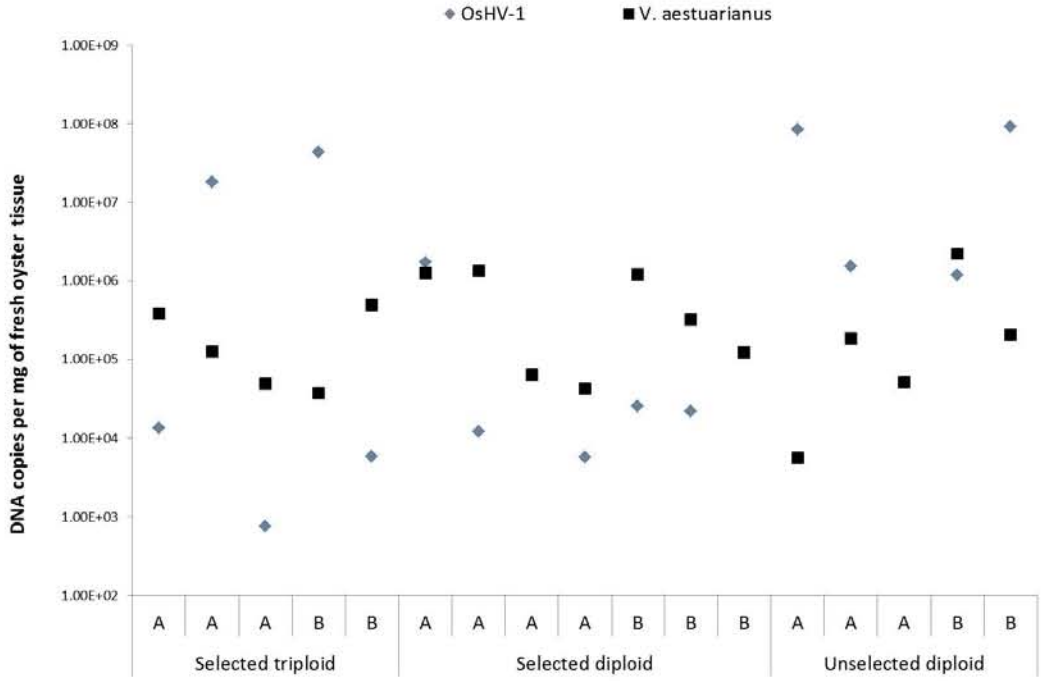

Mortality (%) - V. aestuarianus
100
80
60
40
20
0
Spat1
Spat2
Juvenile
Adult

B

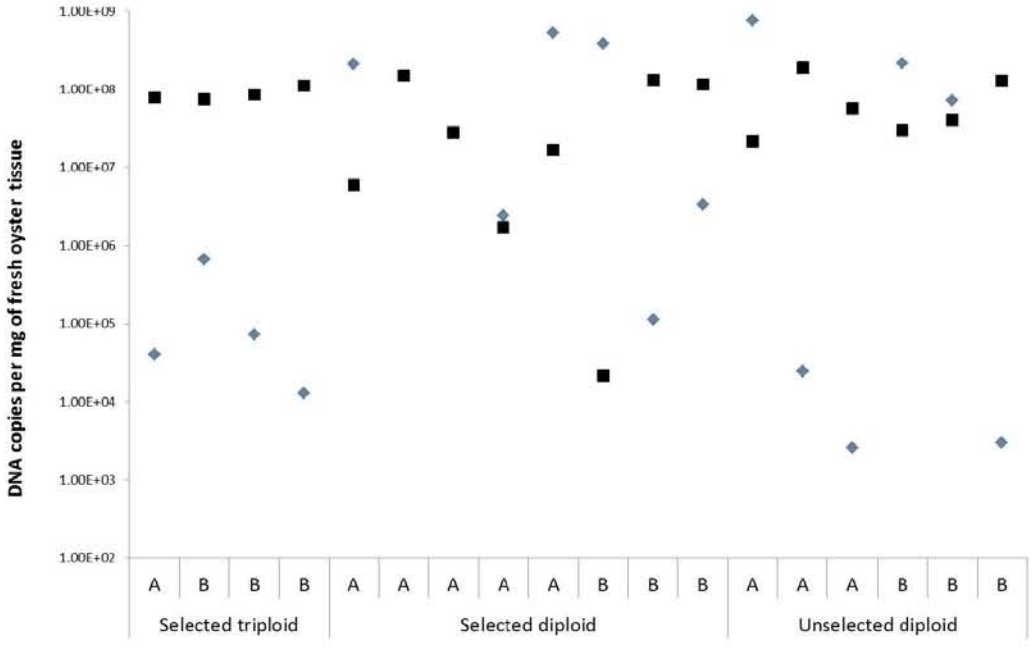

Mortality (%) - dual
100
80
60
40
20
0
Spat1
Spat2
Juvenile
Adult

C

Fig. 2: Mean final mortality (+SD between stocks A and B) of diploid and triploid oysters after either single pathogen exposure to OsHV-1 (A) and *V. aestuarianus* (B) or dual pathogen exposure (C). Trials lasted 15 days for Spat1, Spat2 and Juvenile and one month for Adult. See Table 1 for more details of each trial.



A



B

Fig. 3: DNA copies of OsHV-1 and *V. aestuarianus* detected in individual moribund selected triploid and diploid oysters and unselected diploids after a dual pathogen exposure in Juveniles (A) and Adults (B).

Table 1: Summary of the trials to evaluate OsHV-1 and *V. aestuarianus* susceptibility

Trial	Spat1	Spat2	Juvenile	Adult
Infection protocol	cohabitation	cohabitation	cohabitation	cohabitation
Date of challenge	Jun 2013	Oct 2013	Jun 2014	Mar 2015
Age (months)	4	8	16	25
Individual weight (g)	1.9	3.9	18.4	63.1
Stage	Spat	Spat	Juvenile	Adult
Number of tanks/condition[1]	3	3	3	6
Tank volume (l)	10	10	10	10
Lines $tank^{-1}$	6	6	6	6
Oysters $line^{-1}$ $tank^{-1}$	25	25	15	5

[1] condition: control, OsHV-1 exposure, *V. aestuarianus* exposure, dual pathogen exposure

Table 2: Logit analysis of mortality for selected and unselected lines among diploid oysters for single or dual pathogen exposure

Pathogen	Factor		Spat1		Spat2		Juvenile		Adult	
		DF	F	*p*	F	*p*	F	*p*	F	*p*
OsHV-1	replicate	2	0.93	0.40	3.33	0.04	4.01	0.02	0.91	0.48
	selection	1	80.49	<.0001	107.86	<.0001	24.32	<.0001	0.00	0.98
	stock	1	0.03	0.86	3.7	0.06	0.96	0.33	0.00	0.98
	stock*selection	1	2.68	0.10	1.15	0.28	0.21	0.65	0.00	0.98
Vibrio	replicate	2	5.08	<0.01	25.92	<.0001	5.79	<0.01	1.35	0.25
aestuarianus	selection	1	9.57	<0.01	7.88	<0.01	0.23	0.63	0.01	0.93
	stock	1	0.08	0.77	0.23	0.63	0.77	0.38	1.92	0.17
	stock*selection	1	0.41	0.52	0.32	0.57	0.14	0.71	0.01	0.93
Both	replicate	2	6.70	<0.01	7.60	<0.01	0.39	0.68	0.96	0.44
	selection	1	95.78	<.0001	0.00	0.96	0.00	0.98	5.05	0.03
	stock	1	0.98	0.32	0.00	0.97	0.00	1.00	3.61	0.06
	stock*selection	1	0.22	0.64	0.00	0.98	0.00	1.00	0.41	0.53

stock*selection: interaction between the stocks (A or B) and the selection level (unselected or selected oysters for their higher resistance to OsHV-1)

Table 3: Logit analysis of mortality for triploid and diploid oysters among selected lines for single or dual pathogen exposure

Pathogen	Factor		Spat1		Spat2		Juvenile		Adult	
		DF	F	*p*	F	*p*	F	*p*	F	*p*
OsHV-1	replicate	2	1.19	0.30	1.03	0.36	1.55	0.22	0.00	1.00
	ploidy	1	0.32	0.57	1.37	0.24	0.25	0.62	0.00	1.00
	stock	1	0.00	0.96	0.02	0.89	0.01	0.92	0.00	1.00
	stock*ploidy	1	5.48	0.02	0.48	0.49	0.25	0.62	0.00	0.98
Vibrio	replicate	2	14.45	<.0001	22.49	<.0001	5.53	<0.01	2.01	0.08
aestuarianus	ploidy	1	23.90	<.0001	6.30	0.01	2.25	0.14	0.57	0.45
	stock	1	0.43	0.51	0.19	0.66	6.48	0.01	0.00	0.97
	stock*ploidy	1	2.80	0.10	0.14	0.70	4.40	0.04	2.88	0.09
Both	replicate	2	4.27	0.02	14.03	<.0001	0.83	0.44	0.73	0.60
	ploidy	1	0.02	0.88	7.90	<0.01	0.00	0.97	1.35	0.25
	stock	1	0.43	0.51	0.99	0.32	0.00	0.97	0.37	0.54
	stock*ploidy	1	0.99	0.32	0.01	0.94	0.00	0.98	0.39	0.53

stock*ploidy: interaction between the stocks (A or B) and the ploidy (diploid or triploid oysters selected for their higher resistance to OsHV-1)

12

13 **Table 4: Detection and mean quantification of OsHV-1 and *Vibrio aestuarianus* in Juvenile and Adult stages**

Trial	Pathogen exposure	OsHV-1			*V. aestuarianus*		
		Positive/analyzed	Prevalence (%)	Quantification	Positive/analyzed	Prevalence (%)	Quantification
Juvenile	OsHV-1	9/9	100	$2.8\ 10^{+7}$	3/9	33	$7.7\ 10^{+2}$
	V. aestuarianus	10/25	40	$1.0\ 10^{+3}$	24/25	96	$1.2\ 10^{+6}$
	Both	14/17	82	$1.7\ 10^{+7}$	17/17	100	$4.8\ 10^{+5}$
Adult	OsHV-1	3/3	100	$8.2\ 10^{+8}$	2/3	66	$1.2\ 10^{+5}$
	V. aestuarianus	7/13	54	$3.9\ 10^{+3}$	13/13	100	$1.1\ 10^{+8}$
	Both	16/18	89	$1.4\ 10^{+8}$	18/18	100	$7.1\ 10^{+7}$

Supplementary Table 1 Mortality (%) of the control lines (AC & BC) and the selected lines (AS & BS) after either single pathogen exposure to OsHV-1 and *V. aestuarianus* or dual pathogen exposure

		Spat1		Total spat1	Spat2		Total spat2	Juvenile		Total juvenile	Adult		Total adult
Étiquettes de lignes		Control	Selected		Control	Selected		Control	Selected		Control	Selected	
OsHV-1	**mean**	**90.2**	**33.6**	**61.9**	**79.3**	**6.1**	**42.7**	**60.3**	**14.7**	**37.5**	**17.5**	**1.7**	**8.0**
	A	88.0	40.0	64.0	88.3	7.1	47.7	67.3	16.1	41.7	40.0	0.0	10.0
	B	92.3	27.2	59.7	70.3	5.1	37.7	53.3	13.3	33.3	10.0	3.3	6.7
V. aestuarianus	**mean**	**15.6**	**4.7**	**10.1**	**47.9**	**34.6**	**41.2**	**68.3**	**64.2**	**66.3**	**75.6**	**76.7**	**76.2**
	A	16.0	3.9	9.9	47.6	34.0	40.8	73.3	66.7	70.0	86.7	83.3	84.4
	B	15.1	5.6	10.3	48.2	35.2	41.7	63.3	61.7	62.5	70.0	70.0	70.0
both	**mean**	**90.2**	**22.8**	**56.5**	**98.7**	**75.9**	**87.3**	**100.0**	**90.0**	**95.0**	**60.0**	**78.3**	**70.5**
	A	90.8	26.7	58.8	97.3	72.4	84.8	100.0	93.3	96.7	40.0	73.3	62.2
	B	89.7	18.8	54.2	100.0	79.4	89.7	100.0	86.7	93.3	70.0	83.3	76.7
Total général		**65.3**	**20.4**	**42.8**	**75.3**	**38.9**	**57.1**	**76.2**	**56.3**	**66.3**	**52.3**	**52.2**	**52.3**

AC = line “A” and column “Control”; BC = line “B” and column “Control”
AS = line “A” and column “Selected”; BS = line “B” and column “Selected”

Supplementary Table 2 Mortality (%) of the selected diploid lines (AS & BS) and the selected triploid lines (3nAS & 3nBS) after either single pathogen exposure to OsHV-1 and *V. aestuarianus* or dual pathogen exposure

		Spat1		Total spat1	Spat2		Total spat2	Juvenile		Total juvenile	Adult		Total adult
Étiquettes de lignes		2n	3n		2n	3n		2n	3n		2n	3n	
OsHV-1	**mean**	**33.6**	**30.5**	**32.1**	**6.1**	**9.7**	**7.9**	**14.7**	**11.7**	**13.2**	**1.7**	**3.3**	**2.5**
	AS	40.0	24.5	32.2	7.1	8.6	7.9	16.1	10.0	13.0	0.0	6.7	3.3
	BS	27.2	36.6	31.9	5.1	10.8	8.0	13.3	13.3	13.3	3.3	0.0	1.7
V. aestuarianus	**mean**	**4.7**	**27.6**	**16.2**	**34.6**	**48.9**	**41.7**	**64.2**	**73.9**	**69.0**	**76.7**	**83.3**	**80.0**
	AS	3.9	36.0	19.9	34.0	50.6	42.3	66.7	91.1	78.9	83.3	76.7	80.0
	BS	5.6	19.2	12.4	35.2	47.3	41.2	61.7	56.7	59.2	70.0	90.0	80.0
both	**mean**	**22.8**	**22.3**	**22.5**	**75.9**	**88.5**	**82.2**	**90.0**	**96.7**	**93.3**	**78.3**	**88.3**	**83.3**
	AS	26.7	21.9	24.3	72.4	86.1	79.2	93.3	100.0	96.7	73.3	90.0	81.7
	BS	18.8	22.7	20.7	79.4	90.9	85.2	86.7	93.3	90.0	83.3	86.7	85.0
Total général		**20.4**	**26.8**	**23.6**	**38.9**	**49.0**	**44.0**	**56.3**	**60.7**	**58.5**	**52.2**	**58.3**	**55.3**

AS = line “AS” and column “2n”; BS = line “BS” and column “2n”
3nAS = line “AS” and column “3n”; 3nBS = line “BS” and column “3n”